
Ligand accessibility as means to control cell response to bioactive bilayer membranes

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Abstract: We report a new method to create a biofunctional surface in which the accessibility of a ligand is used as a means to influence the cell behavior. Supported bioactive bilayer membranes were created by Langmuir–Blodgett (LB) deposition of either a pure poly(ethylene glycol) (PEG) lipid, having PEG head groups of various lengths, or 50 mol % binary mixtures of a PEG lipid and a novel collagen-like peptide amphiphile on a hydrophobic surface. The peptide amphiphile contains a peptide synthetically lipidated by covalent linkage to hydrophobic dialkyl tails. The amphiphile head group lengths were determined using neutron reflectivity. Cell adhesion and spreading assays showed that the cell response to the membranes depends on the length difference between head groups of the membrane components.

Cells adhere and spread on mixtures of the peptide amphiphile with the PEG lipids having PEG chains of 120 and 750 molecular weight (MW). In contrast, cells adhered but did not spread on the mixture containing the 2000 MW PEG. Cells did not adhere to any of the pure PEG lipid membranes or to the mixture containing the 5000 MW PEG. Selective masking of a ligand on a surface is one method of controlling the surface bioactivity. © 2000 John Wiley & Sons, Inc. *J Biomed Mater Res*, 50, 75–81, 2000.

Key words: Langmuir–Blodgett; cell adhesion; poly(ethylene glycol); neutron reflectivity; peptide amphiphile; atomic force microscopy

INTRODUCTION

Controlling the response of cells exposed to a biologically active surface, which contains specific functional units, is of increasing practical and scientific interest. Many studies have shown that the physical characteristics of a surface, such as surface topography and chemical composition, can greatly affect how cells respond to a surface.^{1–8} For example, Chen et al.⁹ have shown that the spreading shape and viability of human and bovine capillary endothelial cells can be con-

trolled by changing the size and distribution of islands, adsorbed with extracellular matrix proteins, on micropatterned surfaces. Another example is the use of “intelligent” polymers to change the surface characteristics in response to an environmental stimulus.¹⁰ Such polymers have been used in tissue culture to detach cells from the surface without the need for trypsin.

In this article, we present a way to design a biologically active membrane-like surface in which ligand accessibility is used as a means to control the interaction with cells. We focus on membranes containing a binary mixture of a peptide amphiphile, which has a peptide head group covalently linked to lipid tails,¹¹ and a poly(ethylene glycol) (PEG) lipid. We postulated that the relative height difference between the membrane components determines the accessibility of the peptide ligand to cell surface receptors. When the PEG chains are much shorter than the peptide, the peptide ligand is fully exposed and can be recognized by cells. On the other hand, PEG chains that are much longer than the peptide will cover the ligand completely, resulting in a surface that is inert to cells. By

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using a series of PEG lipids with various PEG chain lengths we are able to control the relative height difference between head groups of the membrane components.

Our initial studies focused on an amphiphile that has a peptide sequence in the head group from the triple helical domain of type IV collagen, known as peptide IVH1. This 15 amino acid peptide (Gly-Val-Lys-Gly-Asp-Lys-Gly-Asn-Pro-Gly-Trp-Pro-Gly-Ala-Pro) is known to play an important role in human and murine melanoma cell adhesion, motility, invasion of basement membrane, and metastasis.¹² A (Gly-Pro-Hyp)₄ repeat [which we will refer to as (GPO)₄] and dialkyl tails are added to this peptide.^{11,13} Circular dichroism (CD) and NMR data showed that the (GPO)₄ repeat and the dialkyl tails induce the otherwise disordered peptide IVH1 to fold into a stable triple helical conformation, the native conformation of the IVH1 peptide in type IV collagen.^{13–15} This conformation is desired because previous studies showed an increase in the biological activity of IVH1 in a triple helical relative to the single stranded form.^{15,16} The dialkyl tails also serve to tether the peptide to a hydrophobic surface via self-assembly or Langmuir–Blodgett deposition.

In contrast to the peptide amphiphile, PEG lipids are effective in preventing protein and cell adhesion to surfaces.^{17,18} Singhvi et al.¹ made self-assembled micropatterned surfaces where the size, shape, number, and distribution of adhesive domains coated with an alkanethiol and nonadhesive domains coated with PEG terminated alkanethiols were used as a means to invoke a specific cell response. Liposomes containing PEG lipids (Stealth™ liposomes, Liposome Technology, Inc.) prolong blood circulating times with half-lives on the order of hours or days compared to conventional liposomes that have a half-life on the order of minutes.^{19,20} The PEG lipids prevent nonspecific cell adhesion to the liposomes and opsonization by plasma proteins. Immuno-stealth liposomes are liposomes that are sterically stabilized by PEG chains and, in addition, have antibodies covalently linked to the PEG chains or the liposome surface.²¹ The antibodies allow specific, antibody mediated targeting of the liposomes against tumors. The ability of immuno-stealth liposomes to work efficiently depends on controlling the relative difference between the distance that the PEG chains and the antibodies extend away from the liposome surface.²²

An adequate design of a membrane-mimetic surface with controlled biological activity requires information about the heights of each of the membrane components. The length of the peptide amphiphile head group, which folds into a triple helical conformation, can be estimated from known X-ray diffraction data.²³ On the other hand, estimation of the height of the PEG lipid head group, using theoretical approximations,

might lead to the wrong conclusions. In a companion article²⁴ we describe a detailed neutron reflectivity study aimed to understand the physical structure of PEG lipids, peptide amphiphiles, and mixed monolayers. In addition, we compare the neutron reflectivity results to theoretical approximations. In the current article, we demonstrate the effectiveness of using ligand accessibility to control the interaction with cells. The results of this study will allow better design of surfaces in which ligand accessibility is used as a means to control the interaction with cells. Furthermore, this approach can be used as the means to locate the active site on a peptide ligand.

MATERIALS AND METHODS

1,2-Distearoyl-*sn*-glycero-3-phosphatidylethanolamine (DSPE) and the PEG lipids used in this study were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). The PEG lipids contained PEG chains, which are covalently linked to DSPE, have average molecular weights (MWs) of 120, 750, 2000, and 5000 and are subsequently referred to as DSPE-PEG-120, DSPE-PEG-750, DSPE-PEG-2000, and DSPE-PEG-5000, respectively. (C₁₆)₂-Glu-C₂-(GPO)₄-IVH1 (Fig. 1; O, hydroxyproline) was synthesized as described elsewhere.¹¹ In the description of the methods that follow we used HPLC grade solvents and deionized water purified in a Milli-Q (Millipore) system to a final resistivity of 18.2 MΩ cm.

The pure amphiphiles were dissolved at approximately 1 mg/mL in a 99:1 chloroform/methanol solution. The solutions were stored at 10°C and heated to room temperature prior to use. Mixtures were made by mixing appropriate amounts of each of the pure component solutions in a 1-mL reaction vial. All the mixed membranes contain 50 mol % of

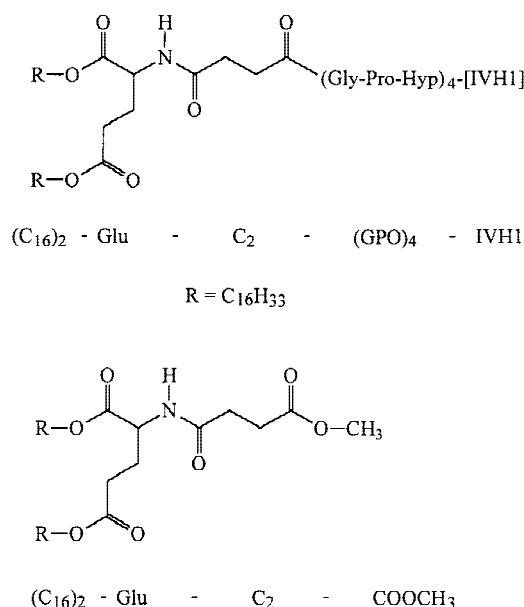


Figure 1. The general structure of the (C₁₆)₂-Glu-C₂-(GPO)₄-IVH1 peptide amphiphile (R = C₁₆H₃₃).

a PEG lipid mixed with the $(C_{16})_2\text{-Glu-C}_2\text{-(GPO)}_4\text{-IVH1}$ amphiphile.

In this study we used the Langmuir–Blodgett (LB) technique to create supported bioactive bilayer membranes. Surface pressure (π) versus molecular area (A) isotherms and the LB film depositions were done on a KSV 5000 LB system (KSV Instruments, Helsinki, Finland). Briefly, prior to use the trough was thoroughly cleaned with a 9:1 chloroform/methanol solution and chromate cleaning solution (Fisher). The clean trough was filled with Milli-Q water and allowed to equilibrate to the desired subphase temperature. After equilibration, an amphiphile solution was spread on the air–water interface with a 100- μL microsyringe (Hamilton). The solvents were allowed to evaporate for 10 min after which the π/A isotherm was recorded. The barrier speed during compression of the layer varied from 5 to 50 mm/min following a program that lowers the speed as the surface pressure increases. The surface pressure was recorded with a flame-cleaned platinum Wilhelmy plate.

Freshly cleaved mica was used as the deposition substrate. Thin 15-mm diameter circular mica disks were cleaved, rinsed with a chloroform/methanol solution following by Milli-Q water, and hung onto the dipper held on edge by clean stainless-steel tweezers. All the depositions were done at a surface pressure (π_{dep}) of 40 mN/m, which is well below the collapse pressure of 60 mN/m. The deposition pressure was held for 10 min prior to deposition to equilibrate the film. The deposition speed for both the up and down strokes was 1 mm/min. The transfer ratio (R_t) was close to one for all depositions. The first step in producing a supported bilayer membrane was to make the mica hydrophobic with a layer of DSPE in the upstroke. The subphase was then discarded, the trough was cleaned, a new subphase was added and equilibrated, and a new layer was spread. After evaporation of the solvent, compression to π_{dep} , and equilibration, the second layer was deposited in the down stroke. The resulting supported bilayer membranes, which if exposed to air rearrange to form monolayers and trilayers, were transferred under water into previously submerged glass vials. The samples were then transferred under water for atomic force microscopy (AFM) characterization or used for cell adhesion assays.

AFM characterization of the LB films was done with a Digital Instruments Nanoscope III system equipped with a fluid cell (Digital Instruments, Santa Barbara, CA). Images were obtained in contact mode under water using Si_3Ni_4 tips (Digital Instruments). A clean, heavier than water, fluid cell o ring was placed on the mica disk substrate. The substrate and disk were then lifted from the water taking care not to disturb the o ring so that a thin water meniscus, held by the o ring, always covered the sample. Excess water outside the o ring was removed, and the sample was placed on a metal disk and positioned on the piezo scanner. The AF microscope head with the fluid cell was then carefully lowered onto the o ring.

M14#5 human melanoma cells were cultured as described elsewhere except that Dulbecco's modified Eagle's medium (DMEM, Celox) was used as the culture media.²⁵ Before the cell adhesion assays the cells were released from culture flasks with 1 mM EDTA in phosphate-buffered saline (PBS, Sigma), washed in adhesion media [DMEM with 10% (v/v) HEPES (Sigma) buffer and 1 mM sodium pyruvate], and

resuspended in adhesion media. In addition, the water in the glass vials holding the LB membranes was changed to the adhesion media and the membranes were heated to 37°C in an incubator. For the adhesion assays, approximately 50,000 cells were added to each glass vial and allowed to adhere for 1 h at 37°C. After 1 h nonadherent cells were removed by washing the membranes 3 times in warm adhesion media. Adherent cells were fixed with a Diff-Quick® stain set (Baxter) that uses methanol to fix cells, eosin Y to stain the cytoplasm, and azure A and methylene blue to stain proteins.

Two criteria were used to quantify the interaction of cells with the supported bilayer membranes. The first is the cell density, which is a measure of cell adhesion, and the second is the shape factor (S) given by the equation

$$S = \frac{4 \pi A}{P^2},$$

where A is the area of the cell and P is the cell perimeter. The shape factor is a measure of the circularity or spreading of cells where circular cells have a shape factor equal to one.

Neutron reflectivity (NR) measurements were carried out on the NG-7 reflectometer at the National Institute of Standards and Technology (NIST, Gaithersburg, MD). The details regarding the experimental procedures and the data analysis are described in detail in a companion article²⁴ and only the minimum essential description is given here. NR measurements of monolayers spread on an *in situ* Langmuir trough were performed at a constant wavelength of $\lambda = 4.8$ Å with a wavelength spread $\Delta\lambda/\lambda$ of about 2.5%. Surface pressure was monitored using a filter paper Wilhelmy plate. A position-sensitive detector, which allows estimation of the intensity from the reading at angles close to the specular angle, was used for the measurements. The analysis of the experimental data typically started with suggestion of a model consisting of several layers, each having a different scattering length density and thickness. To account for surface roughness, the model was convoluted with a Gaussian smearing function.²⁶ The final form of the scattering length density profile was divided into a series of 1 Å thick slabs from which the reflectivity was calculated using the formalism developed by Parratt.²⁷ Best-fit parameters were determined by minimization of the sum of the weighted squared differences between the experimental and the fitted reflectivity so that each experimental point was weighted by its statistical error. Once the parameters were determined the thickness of the layers was extracted from the fitted curves.

RESULTS AND DISCUSSION

The head group length of the $(C_{16})_2\text{-Glu-C}_2\text{-(GPO)}_4\text{-IVH1}$ amphiphile and the PEG lipids that were mixed with the peptide amphiphiles are given in Table I. These values were obtained from the NR recorded at a surface pressure of 40 mN/m (π_{dep}) and room temperature, the same conditions used during the membrane deposition process.²⁴ The head group length of the $(C_{16})_2\text{-Glu-C}_2\text{-(GPO)}_4\text{-IVH1}$ amphiphile is the sum of two components; the $\text{Glu-C}_2\text{-(GPO)}_4$ part and the

TABLE I
Amphiphile Head Group Lengths Obtained from
Neutron Reflectivity Data

Amphiphile	Head Group Length (nm)
(C ₁₆) ₂ -Glu-C ₂ -(GPO) ₄ -IVH1	8.8
DSPE-PEG-120	1.6
DSPE-PEG-750	3.5
DSPE-PEG-2000	9.0
DSPE-PEG-5000	16.8

The head group length of the peptide amphiphile refers to the Glu-C₂-(GPO)₄-IVH1 moiety. The head group length of the PEG lipids is the sum of the lengths of phosphate group, the ethanolamine group, and the PEG chain.

IVH1 sequence. The estimated length of the IVH1 sequence, calculated using 3.0 Å per amino acid in a triple helical conformation, is 45 Å.²³ Thus, the length of the Glu-C₂-(GPO)₄ moiety is approximately 43 Å. The head group length of the PEG lipids is the sum of the lengths of the phosphate group, the ethanolamine group, and the PEG chain. As can be seen from Table I, the head groups of the DSPE-PEG-120 and DSPE-PEG-750 amphiphiles are both shorter than the head group of the (C₁₆)₂-Glu-C₂-(GPO)₄-IVH1 amphiphile where the PEG-750 head group is approximately the same length as the Glu-C₂-(GPO)₄ moiety. The PEG-2000 and the peptide amphiphile head groups have similar lengths, and the PEG-5000 head group is more than twice as long as the peptide amphiphile. The structure of the membranes on the air-water interface and on the solid substrate was assumed to be similar because of the high deposition pressure (40 mN/m) that we used. At this deposition pressure the amphiphiles are in a highly compressed state.

The isotherms of the PEG lipids and the (C₁₆)₂-Glu-C₂-(GPO)₄-IVH1 amphiphile are shown in Figure 2. The DSPE-PEG-120 isotherm shows a small transition at a $\pi = 5$ mN/m. This transition is most likely due to the transition from a liquid expanded (LE) to a liquid condensed (LC) phase.²⁸ The area/molecule of DSPE-PEG-120 at a surface pressure of 40 mN/m (A_{dep}) is 0.4 nm², which is consistent with close packed tails. At the same surface pressure the area/molecule of the (C₁₆)₂-Glu-C₂-(GPO)₄-IVH1 amphiphile is 0.55 nm², which is significantly larger than the cross-sectional area of dialkyl tails. This suggests that the packing density of the (C₁₆)₂-Glu-C₂-(GPO)₄-IVH1 amphiphile is limited by the amphiphile head groups. The DSPE-PEG-750, DSPE-PEG-2000, and DSPE-PEG-5000 isotherms show a large expanded region extending up to the largest area/molecule that we recorded. These amphiphiles have an A_{dep} of 0.55, 0.9, and 1.8 nm², respectively.

AFM micrographs of supported bilayer membranes of the PEG lipids and their mixtures are shown in Figure 3. DSPE-PEG-120 has a smooth surface topol-

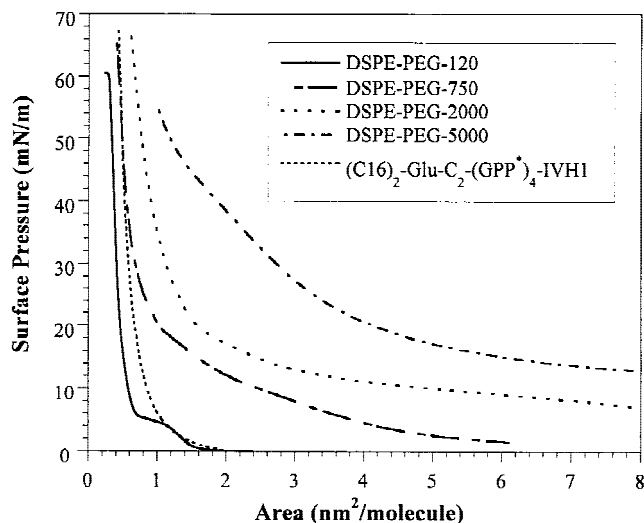


Figure 2. Surface pressure-area isotherms of the PEG lipids and the (C₁₆)₂-Glu-C₂-(GPO)₄-IVH1 amphiphile spread on pure water at 25°C.

ogy with very little defects [Fig. 3(a)]. Conversely, all the other images show defects seen as dark spots. We found that these defects that are present when imaging soft surfaces is likely a result of packing defects that produce soft regions in the membrane. The intensity at which these regions are seen with the AF microscope is related to the force applied by the tip to the sample during imaging. The AF microscope can penetrate or compares the soft regions during imaging; as a result, they appear dark. The density and distribution of the defects on the mixed membranes we are approximately the same in all layers, and we believe they do not play an important role in the membranes bioactivity.

Figure 4 shows images of cells (fixed after a 1-h adhesion assay) on mixed membranes. The results of the quantitative analysis are shown in Figures 5 and 6. Cells do not adhere to membranes of the pure PEG lipids independent of the length of the PEG chain (Fig. 5). Some theoretical approaches suggest that PEG modified surfaces are nonadhesive to proteins due to a steric repulsive force.^{29–31} In addition, Prime and Whitesides³² showed that self-assembled monolayers (SAMs) of poly(ethylene oxide) (PEO) with only two EO segments have the ability to prevent protein adsorption. Clearly our experiments show that there must be a repulsive force, other than the steric repulsive force, that prevents cell adhesion, because cells do not adhere to the DSPE-PEG-120 membrane. This PEG lipid has only three monomer units as the head group. Furthermore, the DSPE-PEG-120 membrane was deposited at a surface pressure of 40 mN/m where the amphiphiles are in the crystalline (LC) phase. Polymer theories do not apply to such short PEG chains and cannot explain the origin of the repulsive force on this membrane. In this case it is possible that the structure

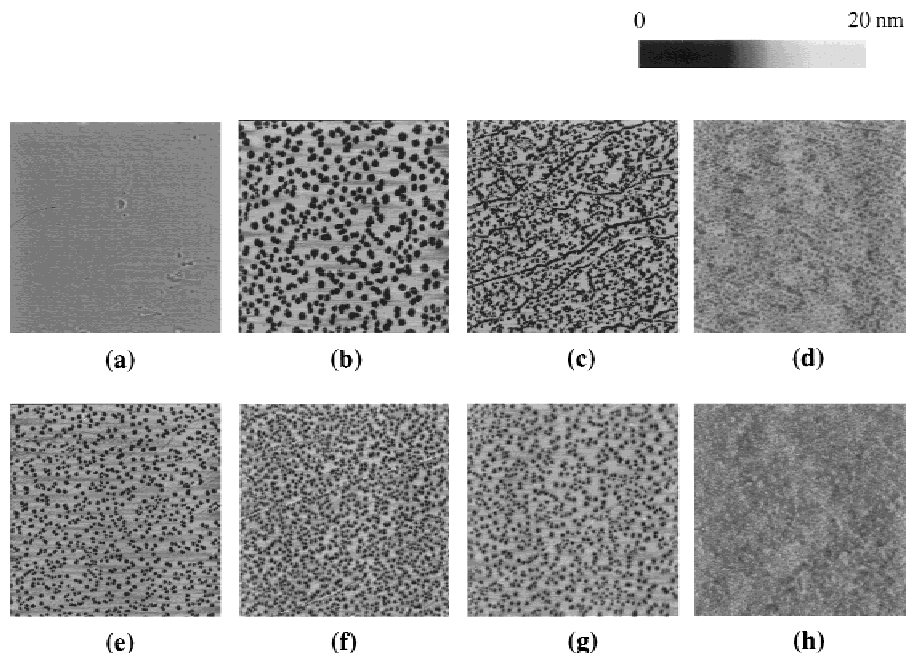


Figure 3. AFM images of the pure PEG lipids: (a) DSPE-PEG-120, (b) DSPE-PEG-750, (c) DSPE-PEG-2000, and (d) DSPE-PEG-5000, and the corresponding 50 mol % $(C_{16})_2$ -Glu- C_2 -(GPO) $_4$ -IVH1 mixture with (e) DSPE-PEG-120, (f) DSPE-PEG-750, (g) DSPE-PEG-2000, and (h) DSPE-PEG-5000. The images are contact mode images of membranes deposited on hydrophobic mica in pure water. Scale bar = 20 μ m.

of the water in the hydration shell above the membrane prevents protein and cell adhesion.

As is the case for the pure PEG lipid membranes, the mixed membrane containing the DSPE-PEG-5000

amphiphile is not adhesive to cells [Fig. 4(d), 5]. In contrast, cells adhere to the DSPE-PEG-120, DSPE-PEG-750, and DSPE-PEG-2000 mixed membranes [Fig. 4(a-c), 5]. The cell density on these membranes is

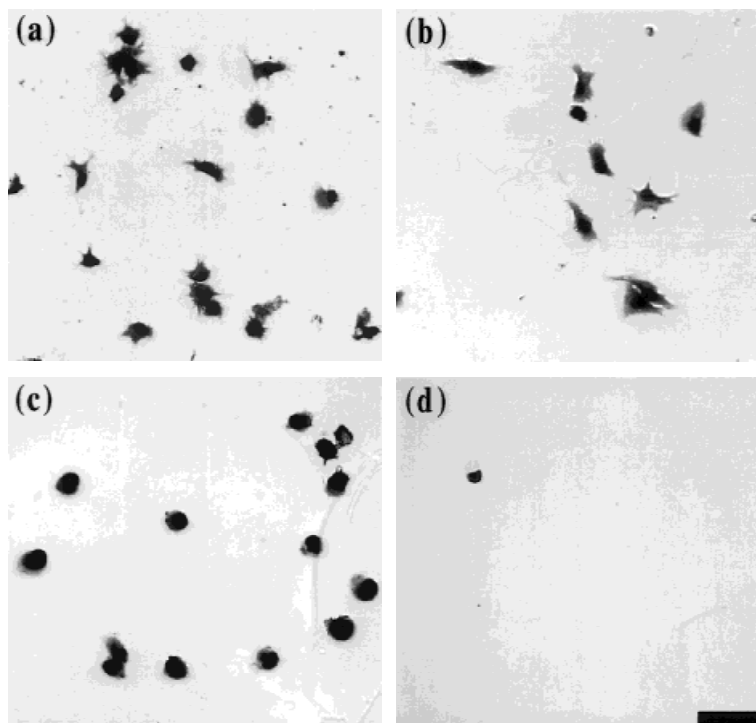


Figure 4. Photomicrographs of M14#5 human melanoma cells fixed after a 1-h adhesion assay on 50 mol % $(C_{16})_2$ -Glu- C_2 -(GPO) $_4$ -IVH1 mixtures with (a) DSPE-PEG-120, (b) DSPE-PEG-750, (c) DSPE-PEG-2000, and (d) DSPE-PEG-5000. Scale bar = 20 μ m.

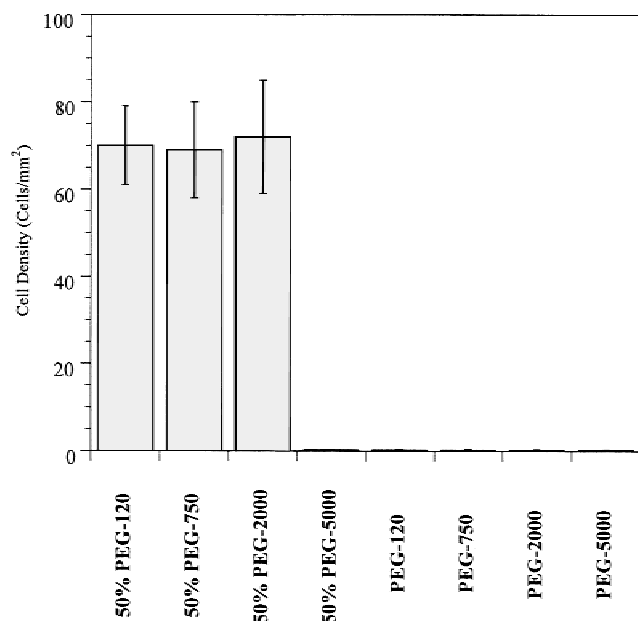


Figure 5. The density of melanoma cells on PEG lipid membranes and on 50 mol % $(C_{16})_2$ -Glu- C_2 -(GPO) $_4$ -IVH1 mixtures. All data represent the means of counting 10 (1 × 1 mm) squares on three different samples.

the same within experimental error (Fig. 5). However, the cells do not spread on the PEG-2000 membrane as indicated by the large number of cells with a shape factor close to one (Fig. 6).

Consequently, by changing the relative length difference between head groups of the membrane components we are able to change the cell response to the membrane-like surfaces in readily observable ways. The length difference between the PEG lipids and the

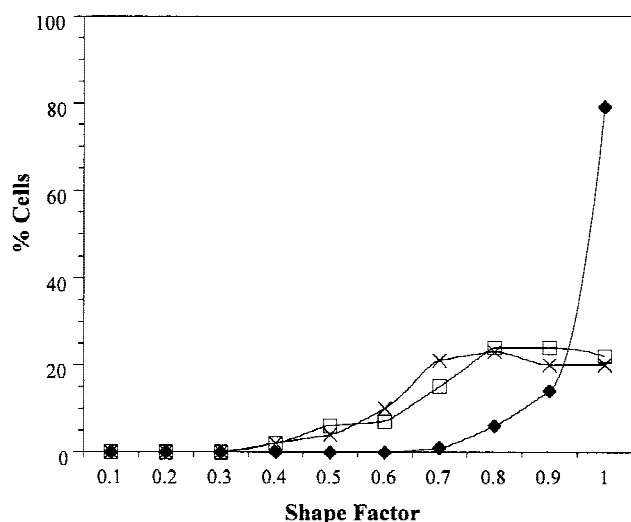


Figure 6. The shape factor of melanoma cells on the 50 mol % $(C_{16})_2$ -Glu- C_2 -(GPO) $_4$ -IVH1 mixtures with (x) DSPE-PEG-120, (□) DSPE-PEG-750, and (◆) DSPE-PEG-2000. All data represent the means of counting 10 (1 × 1 mm) squares on three different samples.

peptide amphiphiles determines the accessibility of the active site of the $(C_{16})_2$ -Glu- C_2 -(GPO) $_4$ -IVH1 amphiphile to the cell surface receptors. Cells adhere and spread on the PEG-120 and PEG-750 mixtures because both these PEG lipids have PEG chains that are much shorter than the head group of the $(C_{16})_2$ -Glu- C_2 -(GPO) $_4$ -IVH1 amphiphile. The PEG chains on the DSPE-PEG-120 and DSPE-PEG-750 amphiphiles only partially mask the (GPO) $_4$ peptide sequence but do not mask the IVH1 peptide sequence. As a result, they do not interfere with the interaction of the cell surface receptors with the IVH1 sequence. Cells adhere but do not spread on the DSPE-PEG 2000 mixture where the 200 MW PEG chain has the same length as the $(C_{16})_2$ -Glu- C_2 -(GPO) $_4$ -IVH1 amphiphile head group.

There are at least three possible explanations for this observation. First, the PEG chains might prevent the clustering of the cell surface receptors, because clustering is required to initiate the signal transduction pathway responsible for cell spreading.¹⁵ Second, it is possible that there are two different signaling sites on the peptide ligand. One site, masked by the PEG chain, is responsible for signaling cell spreading while a second site, near the peptide terminus and not masked by the PEG chains, is responsible for signaling cell adhesion. Prior experiments on the linear form of IVH1 showed that replacement of the three Pro residues by Ala eliminate melanoma cell motility on, but not adhesion to, this sequence.¹² Third, the DSPE-PEG-2000 mixture may have a different surface distribution than the DSPE-PEG-120 and DSPE-PEG-750 mixtures. However, the AFM surface analysis (Fig. 3) shows no obvious differences in distribution among the DSPE-PEG-120, DSPE-PEG-750, and DSPE-PEG-2000 mixtures, making this explanation unlikely.

Cells do not adhere to the EPG-5000 layer because the PEG chain is about twice as long as the $(C_{16})_2$ -Glu- C_2 -(GPO) $_4$ -IVH1 head group. Therefore, the IVH1 peptide is completely masked. In this case the cell only sees the PEG chains when it approaches the surface, similar to what the cell sees when it approaches a surface of the pure PEG lipids.

CONCLUSIONS

We demonstrated that we can manipulate the response of cells to a surface by selectively masking the $(C_{16})_2$ -Glu- C_2 -(GPO) $_4$ -IVH1 amphiphile head group with PEG lipids of varying lengths. By choosing the appropriate PEG lipids and deposition conditions, we showed that we can control the accessibility of a peptide ligand on a surface to cells. As a result, we were able to create three types of surfaces: surfaces that promoted both cell adhesion and spreading, surfaces that promoted cell adhesion but not cell spreading,

and surfaces that did not promote cell adhesion or spreading. Although we used PEG chains of varying lengths to mask the peptide selectively, it is possible to achieve the same result by varying the deposition pressure or grafting density, because this will change the length of the PEG chain but not have a great effect on the length of the $(C_{16})_2$ -Glu- C_2 -(GPO) $_4$ -IVH1 head group.²⁴ In this study we used a specific system with a unique peptide ligand, but we believe that the results of this study will be applicable to other systems and will help in the design of bioactive membranes in which selective masking of a ligand on a surface will be used as a means to control the response of cells.

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